

# Analysis of Methods for Growth Detection in the Search for Extraterrestrial Life

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In the search for life on other planets, experiments designed to detect the growth of microorganisms may prove to be definitive when coupled with chemical characterization and metabolic experiments. If organisms are not abundant, growth provides the only means for obtaining a large mass of biological material suitable for chemical compositional analyses and metabolic assays. Several methods of monitoring growth are described. Of these, optical monitoring in a unique system free of soil particles is advanced as the most appropriate. Theoretical problems related to the formulation of culture media are discussed, and several possible solutions are proposed. The sampling system, the type of monitoring, the size and placement of inoculum, and the medium volume and composition are contingent upon one another and must be integrated without sacrifice to the biological demands.

The methods most commonly utilized for demonstrating the presence of living microorganisms in the soil are growth in liquid nutrient solution, as measured by changes in turbidity, and observation of the development of colonies from soil dusted onto the surface of or immersed in an appropriate solid medium.

The purpose of this paper is to examine how such procedures can be implemented for use in the detection of extraterrestrial life. In addition, other instrumental methods of measuring growth are discussed, and a rationale for the selection of nutrients is developed.

## ROLE OF GROWTH EXPERIMENTS IN THE SEARCH FOR EXTRATERRESTRIAL LIFE

The roles and interrelations of growth and reproduction experiments, chemical composition analyses, and metabolic experiments have been reviewed by Young, Painter, and Johnson (14). These authors assigned a low priority to experiments demonstrating reproduction because of its possible "discontinuous nature and difficulty of implementation . . ." They recommended, however, that experiments attempting to demonstrate growth should be included on a planetary lander because, ". . . the discovery of this attribute would be a very convincing demonstration of the presence of life . . ." In addition, they argue that a variety of positive answers arising from the same sample reinforces the evidence for the presence of life.

Because of the present limitation of analytical

sensitivities, only growth experiments are capable of demonstrating the initial presence of a single viable microorganism. Experiments demonstrating metabolic activities generally require a greater protoplasmic mass, and the number of microorganisms per gram of soil may be too low for the detection of metabolic activity. Thus, amplification by means of a successful growth experiment may be necessary if a planetary lander can collect only small dust samples or if few microorganisms are present.

Growth of many organisms can be demonstrated without any prior knowledge of their metabolic pathways, physiological state, or minimal nutritional requirements. In contrast, the implementation and interpretation of metabolic experiments and chemical analyses require that they be referred to terrestrial organisms or their accumulations. However, should the biochemistry of extraterrestrial organisms differ from that of terrestrial organisms (10), a simple growth experiment could succeed, whereas systems designed to measure specific molecular species might fail because of the potential inapplicability of extrapolating terrestrial biochemistry to the universe as a whole.

## GROWTH MONITORING

Possible and common methods for monitoring the growth of microorganisms are now considered, as well as features which influence the feasibility of these methods for detecting extraterrestrial life by automated instrumentation.

**Gravimetric method.** This method is used frequently for filamentous organisms which do not form homogeneous suspensions suitable for optical monitoring. Although an increase in the dry weight of the particulates is a direct measure of growth, the complexity of processing (collecting, drying, and weighing) is greater than other methods of monitoring. Monitoring is not continuous as measurements are made either from replicate culture tubes or from samples in some sequential order for constructing a growth curve. To achieve sensitivity and dynamic range comparable to other methods, mass would have to be measured in the range from  $10^{-7}$  to  $10^{-4}$  g for 1 ml of suspension, a prohibitive range considering that even sophisticated analytical balances do not have this capability.

**Silting index.** This method was developed recently by a manufacturer of membrane filters and associated equipment (Millipore Corp., Bedford, Mass.) for the assay of particulate matter in a fluid. The method is based upon the principle that particles in a suspension plug the membrane filter pores and decrease the rate of flow of the liquid through the filter. In order to work with a small volume of a suspension, we modified this method by first depositing a suspension of bacterial cells on a filter (pore size,  $0.45 \mu$ ) and then recording the time required to push 20 ml of distilled water through the filter (Fig. 1). The total cross-sectional areas of the cells (*Sarcina lutea*) at the concentrations which resulted in proportional increases in filtration time were calculated to be within 15 to 75% of the calculated filter pore area. The particulate concentration range measurable with this method can be changed by varying the filter area, the volume of fluid, or the applied pressure. No washing and drying of the particulates are required, but monitoring cannot be continuous.

**Electronic particle counting.** The Coulter counter (Coulter Electronics Co., Hialeah, Fla.) is most commonly used in counting red blood cells. Although bacterial cells can be counted and their volume determined with this instrument, the required  $30\text{-}\mu$  orifice is easily clogged. Changes in the conductivity of the solution may also affect the detectability of the particles. Because of these factors, this method appears to be unsuitable.

**Metabolic.** Metabolic monitoring, i.e., rate of substrate consumed, rate of products produced, changes in pH and redox potential, is used as a means of following growth of microorganisms. This technique is not direct, but inferential. Levin and Heim (6) suggested that growth can be demonstrated by the evolution of  $^{14}\text{CO}_2$ , from a labeled substrate, at a logarithmic rate corre-

sponding to the growth rate of bacteria. However, organisms can also grow in accordance with linear or square-root rates, and it appears to be an unnecessary restraint to be required to predict the growth kinetics in order to be able to demonstrate growth. The deduction of growth from the rate of production of products should be limited to well-known systems with no alternate pathways (7).

**Chemical composition of particles.** If organisms are growing in a nutrient solution, sequential analysis of samples washed free of soluble materials will contain increasing amounts of organic carbon and Kjeldahl nitrogen. Methods for such automated analyses of growing cultures are available (1). Although such analyses of the particulate matter are considered essential for verifying the biochemical composition, we consider them to be a suitable course of action when the particles have attained a concentration which appears to be within the range of sensitivity of the specific chemical determination. This permits a correlation between the concentration of particulates and the concentration of specific organics. A discussion of specific chemical tests is outside the scope of this paper.

**Optical techniques applied to a surface.** The growth of microorganisms, in particular, the fungi, is frequently monitored by measuring the changes in diameter of a colony on an agar surface. This method can be automated (2), and we have used it (Fig. 2) in detecting growth of bacterial colonies from desert soil particles sprinkled on an agar plate. This technique is attractive because it makes possible a high ratio of soil to medium. An opaque substrate, such as a membrane filter, could be used with a reflected dark-field optical system. However, the information storage capacity or transmittance requirements are very high, and it may be difficult to separate the new particles (growth) from the substrate for further characterization and analysis.

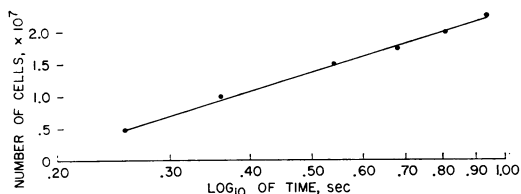


FIG. 1. Number of bacterial cells versus time for fluid flow. Cells of *Sarcina lutea* deposited on a  $0.45\text{-}\mu$  Millipore filter in a circular area (1 cm in diameter). The time measured includes the total time required to push the 20 ml of distilled water through the filter with the 12.3-kg weight using the Millipore silting index apparatus.

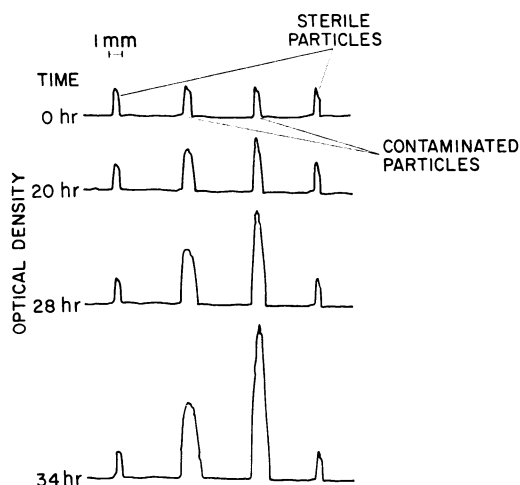


FIG. 2. Optical density changes at desert sand particles incubated on a nutrient agar plate. The same four particles were scanned repeatedly with the Joyce-Loeble microdensitometer.

Furthermore, plate counts yield fewer soil microorganisms than the most probable number dilution method, according to Krasnil'nikov (5). The reason for this is not understood, but it may be caused by the separation of synergistic microorganisms, or by limitations imposed by establishing only one atmospheric composition for all organisms, or by inadequate separation of microorganisms on the surface.

**Optical techniques in a liquid.** Increase in optical density is probably the most common laboratory procedure for monitoring the growth of bacteria. Light may be absorbed or scattered by cells, and the relative importance of these two phenomena depends upon the wavelength of light, the geometry of the instrument, and the composition of the cells and the suspending medium. Techniques which measure only scattered light are more sensitive than techniques which include unscattered light as well. Vishniac proposed light-scattering instrumentation for space application in detecting growth in liquid media (11). Light-scattering measurements are not independent of light absorbance; the path length through the medium changes with the amount of scattering, and the amount of scattered light detected depends upon the absorbance of the medium. Although the problem of optical monitoring for particulate matter only has not been resolved, other phenomena associated with introducing soil particles into a liquid nutrient may lead to much greater ambiguities.

With certain soils, the following may occur.

(i) Soil high in clay content may form a gel when

wet. (ii) Dilute suspensions of clay-size particles can scatter more light than a concentration of  $10^7$  bacteria per ml. (iii) Growth of microorganisms may be accompanied by the production of adhesives which bind soil particles; this is a well-known phenomenon in soil microbiology and can be accompanied by a decrease in optical density. (iv) Most of the growth may take place either at the gas-liquid interface or in association with the soil particles at the bottom of the culture vessel or cuvette. Stirring or mixing a suspension would be required to obtain meaningful optical-density readings, but stirring would, at the same time, preclude the use of high soil concentrations in the medium.

In order to resolve this dilemma, two modified methods were examined. The first method consisted of incubating soil particles with a liquid medium without attempting to achieve an initial low optical density. At intervals, the suspension was diluted with fresh medium. Thus, the proportion of particles which reproduced increased while the original inorganic particles were diluted out. The particles which reproduced eventually produced the dominant signal (Fig. 3).

It is possible to estimate the number of 1:2 dilutions required to reduce optical densities (1-cm path length) of small interfering particles

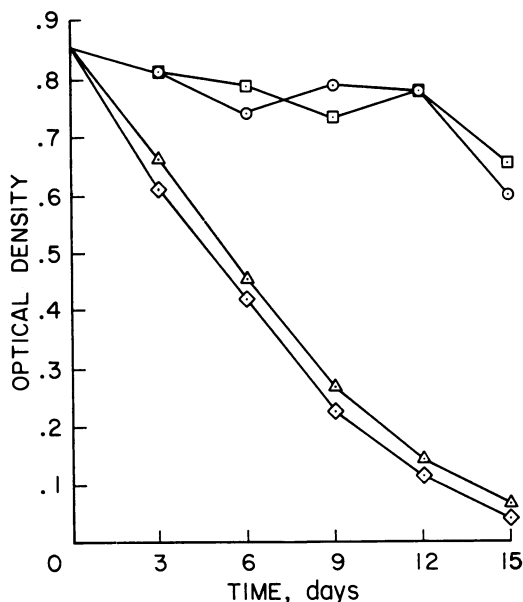


FIG. 3. Repetitive dilution technique with soil incubated in water,  $\Delta$ ; salt solution,  $\diamond$ ; Czapek-Dox broth,  $\circ$ ; and Trypticase Soy Broth (BBL),  $\square$ . The soil suspension was diluted every 3rd day (1:2) immediately after the optical density (525  $m\mu$ ) was read with a Spectronic-20 colorimeter.

suspended in a solution:

*Given*

Density of particle, 1.2 g/cc

$10^5$  particles ( $1\ \mu$  in diameter) per cc would result in an acceptable signal from the soil particles (ca. electronic noise levels of spectrophotometer)

Initial concentration of slurry = 0.6 g/ml

*Then*

$$10^5 = \text{initial number of particles} \times \frac{1}{2}^N$$

$$= \frac{0.6\ \text{g}}{\frac{1.2\ \text{g/cc}}{1/6\pi \times 10^{-12}}} \times \frac{1}{2}^N$$

$$= 10^{12} \times \frac{1}{2}^N$$

$$2^N = 10^7$$

$$N \log 2 = 7 \times \log 10$$

$$N = 23$$

Thus, twenty-three 1:2 dilutions would be necessary to eliminate interference from soil particles. With a fluid volume of 1 cc, only 11.5 cc of additional fluid is needed. This method requires (i) that the initial ratio of soil to medium not form a gel, and (ii) that the growth rate be greater than the dilution rate.

The second method requires a two-level

chamber with a sintered-glass plate forming the floor of the upper level and dipping into a liquid in the lower level. The fluid in the lower level is monitored by a suitable optical method. Figure 4 illustrates a model of such a chamber. A dry soil sample is dispensed into the upper level, and a liquid medium is dispensed into the lower level. Due to the capillary structure, the liquid rises in the sintered-glass plate and wets the base of the soil, and the soil due to its own physical nature wets to its maximum water-holding capacity. Additional fluid is then added to the lower level to compensate for the fluid absorbed by the soil and to maintain a level above the light path. Microorganisms will migrate or grow from the soil into the medium in the lower level. With this technique, the physical parameters of the soil need not be defined, and the growth medium can be monitored without soil particulate interference (Fig. 5). Additions to the medium can be made any time, or the medium replaced without requiring a new sample. A stirring bar or vibrator can be included in the lower level for dispersing the particles before each reading. Additional advantages are that metabolic experiments, such as soil gas exchange, can easily be coupled to this chamber, and particles free of contaminating soil will be available for chemical analysis.

Both of these methods require substantial growth for detection purposes since neither method is capable of detecting the initial growth. This was not considered to be a serious drawback for two reasons: (i) the first concern is to provide

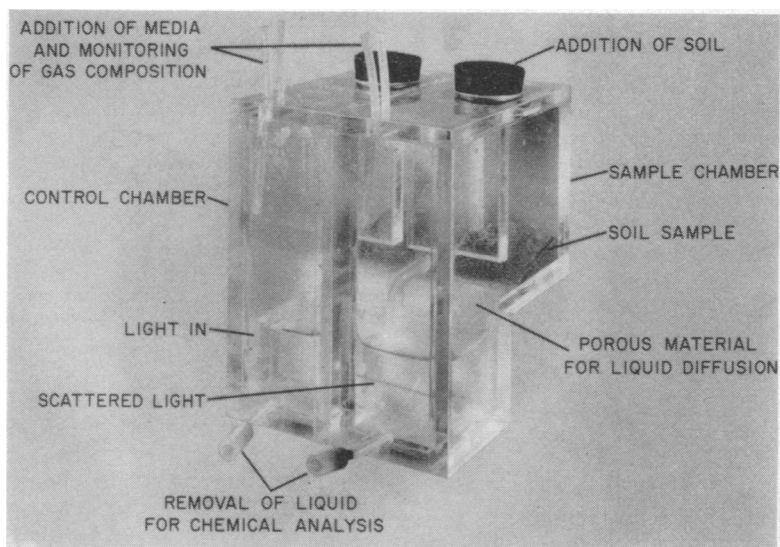


FIG. 4. Models of the two-level growth chamber used for growth monitoring by light scattering. In the right chamber, the light of a laser beam is scattered by particles in suspension.

an environment suitable for growth without regard to the sensitivity, for without growth the sensitivity has little value; and (ii) substantial growth, free from soil particulates, assures a larger sample for chemical characterization with increased confidence in the organic nature of the particulates.

#### MEDIA

When searching for unknown microorganisms, it is tempting to devise conditions favorable for the growth of photoautotrophic organisms because of minimal nutritional requirements. Their energy source is solar, and their carbon requirement is met by atmospheric carbon dioxide. In nature, the growth of autotrophs is also accompanied by the growth of heterotrophs. However, in considering the probable evolutionary sequence of nutritional forms (8), there is some justification in believing that heterotrophic organisms would always be present, but autotrophic organisms may not (4). Although Sagan (12) has written "such an *exclusively heterotrophic* system is inherently limited by the total free energy available in the organic medium, and organisms arising in such an environment can be expected, after an initial geometric increase in numbers, to die of malnutrition," there are reasons to believe that organic compounds may be formed on planetary surfaces even today (15), thus providing continuing supplies of nutrients for heterotrophs.

The following discussion will consider only heterotrophic microorganisms. The first section considers bulk soil acquisition from a planetary surface, the second section an initial enrichment *in situ* prior to a small sample acquisition.

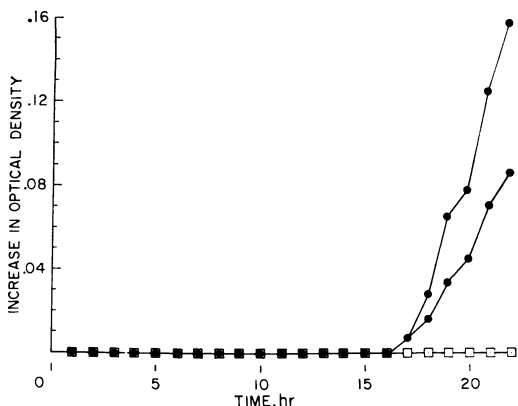


FIG. 5. Changes in turbidity in lower level of growth chamber. Upper level inoculated with 0.5 g of desert sand (●), or sterilized desert sand (□). A 1-ml amount of Trypticase Soy Broth injected into lower chamber. Initial density (tungsten lamp, Jouan Biophotometer) of 0.01.

**Bulk samples.** The advantages of a high ratio of soil to medium stem from two assumptions: (i) the larger the soil sample, the greater the probability of obtaining a viable organism in the growth chamber; and (ii) the sample itself may provide the micronutrients and adjust the medium toward local ionic distributions and concentrations.

Since many organisms which appear to be indigenous to extreme environments will grow faster in the laboratory if no attempt is made to simulate the environment, an argument can be developed against such simulation. However, we believe there is a probability of killing the organisms adapted to the indigenous environment by any sudden or drastic change. Weston (13) discussed some of the limitations inherent in attempting to simulate the environment by preparing a nutrient solution according to the information obtained by a soil chemical analysis. Consequently, we propose that the test sample itself be used for conditioning the medium to local conditions.

The proper temperature range for growth can be assumed to be those of the planet. Since there is no reason to believe that liquids other than water can provide the solvent for life (4), the temperature must be within the limits permitting the existence of liquid water under ambient conditions. Since any indigenous soil microorganisms would not be growing in pure water, but in a salt solution in soil, the upper and lower limits of temperature wherein liquid water exists would be unknown until the soil solution was characterized. Microorganisms can grow below 0°C (for a pertinent compilation of the literature, see reference 9). For Martian exploration, for example, it would appear necessary to be able to maintain temperatures at least between -30 to 20°C.

Water activity must be suitable for the indigenous microorganisms. Since active indigenous organisms would necessarily be capable of growing in the native soil solution, it would seem appropriate to adjust the nutrient medium accordingly. Either the indigenous soil solution itself could be used, or appropriate adjustment could be made by incubating a large quantity of planetary surface material with a small volume of nutrient medium. This would also provide unpredicted, but potentially essential, cations and anions and help adjust the medium to native H<sup>+</sup> concentration. The results presented in Table 1 indicate that the use of the two-level growth chamber, with an unbuffered medium, may help provide for such an adjustment.

In addition to adjusting water activity, the soil can provide other nutrients for the growth of

TABLE 1. *pH values of soil<sup>a</sup> and pH values of 1 ml of water in lower level with 0.5 g of soil in upper level of two-level growth chamber*

Soil number	Soil paste	Water in lower level
1	2.9	3.2
2	3.1	3.4
3	4.6	5.6
4	6.6	6.5
5	7.3	7.6
6	7.7	7.1
7	7.8	7.4
8	8.1	7.1
9	8.8	7.7

<sup>a</sup> Soil paste pH.

indigenous microorganisms. We can assume that, if there are growing organisms in the soil of another planet, the nutrients are already there.

Another assumption is that, given a planetary sample, an appropriate extract can be made. In preliminary experiments, both the soil solution and neutralized sodium hydroxide extracts of a saline soil containing 1% organic carbon supported the growth of at least some indigenous microorganisms (Table 2). Both replenishing the soil solution and using sodium hydroxide extracts as media increased the total growth over that obtained in the soil solution. Consequently, it is possible to visualize procedures that will permit detectable growth to be attained either by replenishing a dilute medium such as the soil solution, by extracting in such a manner that nutrients are concentrated, or by concentrating a dilute extract of the soil. Although the availability of nutrients in nonrhizospheric desert soils has yet to be investigated extensively, the number of heterotrophic microorganisms in two representative dry desert soils increased with the addition of water (Table 3). This increase occurred within the first 2 days of incubation, and the viable population remained constant for at least another 17 days. The increase of only  $3 \times 10^5$  cells per g of soil B, however, suggests that the readily available nutrients would have to be concentrated from at least 100 g of soil to obtain substantial growth in 1 ml of fluid. This concentration must be achieved without a concurrent concentration of salts. The population which grew in the incubated soil was not representative of the dominant population before incubation (Table 4). Either successional factors were operative in permitting a previously minor component of the population to gain dominance, or the initial dominant population was a result of its survival ability rather than its competitiveness. In any case, the change in the population suggests that the

organisms able to grow best in an extract medium would not be the most numerous organisms present. Therefore, a preliminary incubation of the soil inoculum with water, before incubation in the extract medium, might supply a more effective inoculum.

The growth chamber atmosphere could be similar to the prevailing planetary atmosphere or similar to the gas composition of the subsurface. Although the introduction of gases not indigenous to the environment could be toxic to the major population, all known components of the planetary atmospheres, except for the noble gases, can be utilized in some phase by various terrestrial microorganisms. Consequently, the addition of  $N_2$ ,  $NH_3$ , or  $CH_4$  could provide carbon and nitrogen, and  $CO_2$  and  $O_2$  could supply the electron acceptors for heterotrophic microorganisms.

TABLE 2. *Increase in viable count per milliliter after incubating soil with water or soil extracts<sup>a</sup>*

Incubation solution	Increase in viable count
Controls	
Distilled water.....	133
Distilled water replenished.....	145
Soil solution.....	$2.4 \times 10^6$
Soil solution replenished.....	$1.1 \times 10^8$
NaOH extract.....	$3.9 \times 10^7$

<sup>a</sup> A 0.1-mg amount of Bowers clay, obtained by dilution, was incubated with 4 ml of each solution. From colony counts of this soil on Trypticase Soy Broth (BBL) medium and Czapek-Dox medium, these suspensions were calculated to contain approximately 25 organisms per ml, which would form colonies on these media, at the beginning of each incubation period. The increases in the viable counts reported above were made after a 28-day incubation period, except for the NaOH extract which was terminated in 9 days. (Other experiments indicated that continued incubation in the NaOH extract would not have increased the viable count substantially and that most of the organic matter removed by the NaOH extraction is not readily available to microorganisms.) Soil solutions were obtained by vacuum filtration of a paste made from 70 g of air-dried Bowers clay and 50 ml of water. Replenishing, where indicated, was accomplished by centrifugation of the particles and microorganisms, removal of 2 ml of the supernatant fluid, and addition of 2 ml of water or the soil solution. This was done after 4, 10, 12, 21, and 25 days of incubation. The NaOH extract was prepared by extracting 500 g of Bowers clay with 1,500 ml of 0.25 NaOH for 2 hr at 85°C and passing the filtrate through an Amberlite XE-89 ( $H^+$ ) column and eluting with 1 N NaOH to bring the filtrate up to a soil pH of 7. Sterilization was accomplished by filtering the solutions through an ultrafine glass filter.

TABLE 3. Increase in viable cell count per gram<sup>a</sup> of desert soil wetted to maximal water-holding capacity

Incubation solution	Increase	
	Soil A	Soil B
Water.....	$8 \times 10^6$	$0.3 \times 10^6$
Glucose solution, 1%...	$20 \times 10^6$	$4 \times 10^6$
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> solution, 0.1%.....	$2 \times 10^6$	$0.3 \times 10^6$

<sup>a</sup> Initial counts were  $3 \times 10^5$  (A) and  $4 \times 10^3$  (B)

TABLE 4. Per cent morphological types of bacteria before and after incubation of soil with water

Morphological type	Soil A		Soil B	
	Before	After	Before	After
Sporeforming rods....	2	10	90	0
Actinomycetes.....	95	0	5	0
Cocci.....	3	65	5	35
Pleomorphic rods....	0	25	0	10
Small motile rods....	0	0	0	55

*Enriched samples in situ.* To detect small numbers of organisms capable of growing on a selected medium, an enrichment procedure could be used. Thus, if a medium were slowly dispensed on a protected area of a planetary surface, some indigenous organisms might be present which could utilize this medium. These organisms should increase and be more readily detectable if a small sample of the surface were later incubated in the same medium.

This principle was tested by use of sand from Death Valley, Calif. (Fig. 6). The nutrients were dripped on a protected sample for 1 week; then the sand particles from the surface were spread in a sterile petri dish and allowed to air-dry. One grain of sand was then dropped into each of three tubes containing 9 ml of the same medium. Three tubes of the same broth were also inoculated with one grain of sand which had not been previously incubated with the medium. The data demonstrate striking differences in the inocula.

This method may also permit some adaptation of indigenous organisms *in situ* to the specific nutrients, and the nutrient components may not be as critical as it now appears. Some rationale, however, must be considered in formulating the media.

A variety of media should be included in early missions for detecting viable organisms from extraterrestrial samples. From our current

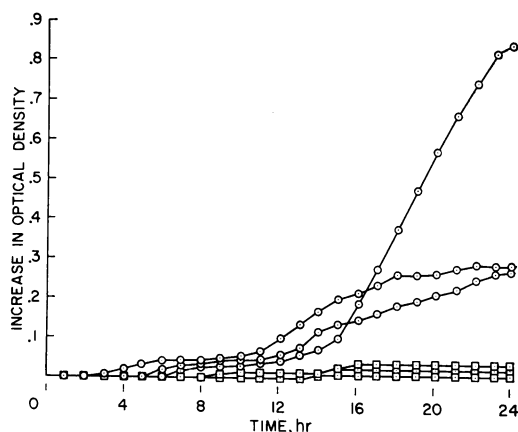


FIG. 6. Changes in optical density of nutrient media inoculated with single sand grains. Sand preincubated with medium,  $\circ$ , sand not preincubated with medium,  $\square$ .

knowledge of microorganisms on the Earth, we can select nutritionally adequate media to grow a wide selection, but is this sufficient?

Various biologically significant organic compounds have been synthesized in reducing atmospheres which simulate the possible primary Earth atmosphere or the present or primary atmosphere of other planets. Horowitz (3) suggests "the substrates most likely to be metabolized by Martian life are simple organic compounds like those produced by the irradiation of simulated primitive atmospheres." Our experiments have so far indicated that one-half of the organisms which will form colonies on glucose-ammonium chloride-mineral salts medium will also form colonies on the same medium with glucose replaced by products (formal sugars) obtained by the condensation of formaldehyde. Therefore, it is suggested that it is appropriate to include media developed according to what is known of the relative abundance of elements, the products of abiogenic synthesis, or, as suggested by the analysis of carbonaceous chondrites, such media should be relatively free from the bias stemming from our familiarity with the organisms on Earth.

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